MULTIPLE DRUG RESISTANCE GENE atrD OF *ASPERGILLUS NIDULANS*

Technical Field of the Invention

This invention relates to recombinant DNA technology. In particular, the invention concerns the cloning of nucleic acid encoding a multiple drug resistance protein of *Aspergillus nidulans*.

Background of the Invention

Multiple drug resistance (MDR) mediated by the human *mdr*-1 gene product was initially recognized during the course of developing regimens for cancer chemotherapy (Fojo *et al.*, 1987, *Journal of Clinical Oncology* 5:1922-1927). A multiple drug resistant cancer cell line exhibits resistance to high levels of a large variety of cytotoxic compounds. Frequently these cytotoxic compounds will have no common structural features nor will they interact with a common target within the cell. Resistance to these cytotoxic agents is mediated by an outward directed, ATP-dependent pump encoded by the *mdr*-1 gene. By this mechanism, toxic levels of a particular cytotoxic compound are not allowed to accumulate within the cell.

MDR-like genes have been identified in a number of divergent organisms including numerous bacterial species,

the fruit fly *Drosophila melanogaster*, *Plasmodium falciparum*, the yeast *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Leishmania donovanii*, marine sponges, the plant *Arabidopsis thaliana*, as well as *Homo sapiens*. Extensive searches have revealed several classes of compounds that are able to reverse the MDR phenotype of multiple drug resistant human cancer cell lines rendering them susceptible to the effects of cytotoxic compounds. These compounds, referred to herein as "MDR inhibitors", include for example, calcium channel blockers, anti-arrhythmics, antihypertensives, antibiotics, antihistamines, immuno-suppressants, steroid hormones, modified steroids, lipophilic cations, diterpenes, detergents, antidepressants, and antipsychotics (Gottesman and Pastan, 1993, *Annual Review of Biochemistry* 62:385-427). Clinical application of human MDR inhibitors to cancer chemotherapy has become an area of intensive focus for research.

On another front, the discovery and development of antifungal compounds for specific fungal species has also met with some degree of success. *Candida* species represent the majority of fungal infections, and screens for new antifungal compounds have been designed to discover anti-*Candida* compounds. During development of antifungal agents, activity has generally been optimized based on activity against *Candida albicans*. As a consequence, these anti-*Candida* compounds frequently do not possess clinically significant activity against other fungal species such as *Aspergillus nidulans*. However, it is interesting to note that at higher concentrations some anti-*Candida* compounds are able to kill other fungal species such as *A. nidulans* and *A. fumigatus*. This type of observation suggests that the antifungal target(s) of these anti-*Candida* compounds is present in *A. nidulans* as well. Such results indicate that *A. nidulans* may possess a natural mechanism of resistance that permits them to survive in clinically relevant

concentrations of antifungal compounds. Until the present invention, such a general mechanism of resistance to antifungal compounds in *A. nidulans* has remained undescribed.

Summary of the Invention

The invention provides, *inter alia*, isolated nucleic acid molecules that comprise nucleic acid encoding a multiple drug resistance protein from *Aspergillus nidulans*, herein referred to as atrD, vectors encoding atrD, and host cells transformed with these vectors.

In another embodiment, the invention provides a method for determining the fungal MDR inhibition activity of a compound which comprises:

a) placing a culture of fungal cells, transformed with a vector capable of expressing atrD, in the presence of:

(i) an antifungal agent to which said fungal cell is resistant, but to which said fungal cell is sensitive in its untransformed state;

(ii) a compound suspected of possessing fungal MDR inhibition activity; and

b) determining the fungal MDR inhibition activity of said compound by measuring the ability of the antifungal agent to inhibit the growth of said fungal cell.

In still another embodiment the present invention relates to strains of *A. nidulans* in which the atrD gene is disrupted or otherwise mutated such that the atrD protein is not produced in said strains.

In yet another embodiment, the present invention relates to a method for identifiying new antifungal compounds comprising the use of atrD$^-$ gene disruption or gene replacement strains of *A. nidulans*.

Detailed Description of the Invention

The present invention provides isolated nucleic acid molecules that comprise a nucleic acid sequence encoding atrD. The cDNA (complementary deoxyribonucleic acid) sequence encoding atrD is provided in the Sequence Listing as SEQ ID NO: 1. The amino acid sequence of the protein encoded by atrD is provided in the Sequence Listing as SEQ ID NO: 2.

Those skilled in the art will recognize that the degenerate nature of the genetic code enables one to construct many different nucleic acid sequences that encode the amino acid sequence of SEQ ID NO: 2. The cDNA sequence depicted by SEQ ID NO: 1 is only one of many possible atrD-encoding sequences. Consequently, the constructions described below and in the accompanying examples for the preferred nucleic acid molecules, vectors, and transformants of the invention are illustrative and are not intended to limit the scope of the invention.

All nucleotide and amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. §1.822(b)(1994).

The term "vector" refers to any autonomously replicating or integrating agent, including but not limited to plasmids, cosmids, and viruses (including phage), comprising a nucleic acid molecule to which one or more additional nucleic acid molecules can be added. Included in the definition of "vector" is the term "expression vector". Vectors are used either to amplify and/or to express deoxyribonucleic acid (DNA), either genomic or cDNA, or RNA (ribonucleic acid) which encodes atrD, or to amplify DNA or RNA that hybridizes with DNA or RNA encoding atrD.

The term "expression vector" refers to vectors which comprise a transcriptional promoter (hereinafter "promoter") and other regulatory sequences positioned to drive

expression of a DNA segment that encodes atrD. Expression vectors of the present invention are replicable DNA constructs in which a DNA sequence encoding atrD is operably linked to suitable control sequences capable of effecting the expression of atrD in a suitable host. Such control sequences include a promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control termination of transcription and translation. DNA regions are operably linked when they are functionally related to each other. For example, a promoter is operably linked to a DNA coding sequence if it controls the transcription of the sequence, or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The term "MDR inhibition activity" refers to the ability of a compound to inhibit the MDR activity of a host cell, thereby increasing the antifungal activity of an antifungal compound against said host cell.

In the present invention, atrD may be synthesized by host cells transformed with vectors that provide for the expression of DNA encoding atrD. The DNA encoding atrD may be the natural sequence or a synthetic sequence or a combination of both ("semi-synthetic sequence"). The *in vitro* or *in vivo* transcription and translation of these sequences results in the production of atrD. Synthetic and semi-synthetic sequences encoding atrD may be constructed by techniques well known in the art. *See* Brown et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., 68:109-151. atrD-encoding DNA, or portions thereof, may be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A,380B, 394 or 3948 DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404).

Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of nucleic acid sequences may be constructed which encode atrD. All such nucleic acid sequences are provided by the present invention. These sequences can be prepared by a variety of methods and, therefore, the invention is not limited to any particular preparation means. The nucleic acid sequences of the invention can be produced by a number of procedures, including DNA synthesis, cDNA cloning, genomic cloning, polymerase chain reaction (PCR) technology, or a combination of these approaches. These and other techniques are described by Maniatis, *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), or *Current Protocols in Molecular Biology* (F. M. Ausubel *et al.*, 1989 and supplements). The contents of both of these references are incorporated herein by reference.

In another aspect, this invention provides the cDNA encoding atrD, which may be obtained by synthesizing the desired portion of SEQ ID NO:1 or by following the procedure carried out by Applicants. This procedure involved construction of a cosmid genomic DNA library from *Aspergillus nidulans* strain OC-1, a mutant derived from A42355. This library was screened for genes related to MDRs using a homologous probe generated by PCR. Degenerate PCR primers directed towards amplification of DNA sequences encoding highly conserved regions found in the ATP-binding domain of several MDR genes were synthesized. PCR using these primers and *Aspergillus nidulans* genomic DNA as template produced an approximately 400 base pair DNA fragment. The DNA sequence of this fragment was highly homologous to the ATP-binding region of several MDRs as predicted. This fragment was used as a hybridization probe to identify cosmid clones containing the entire atrD gene. A

subclone from one such cosmid containing the entire atrD gene was sequenced to ascertain the entire sequence of atrD.

To effect the translation of atrD-encoding mRNA, one inserts the natural, synthetic, or semi-synthetic atrD-encoding DNA sequence into any of a large number of appropriate expression vectors through the use of appropriate restriction endonucleases and DNA ligases. Synthetic and semi-synthetic atrD-encoding DNA sequences can be designed, and natural atrD-encoding nucleic acid can be modified, to possess restriction endonuclease cleavage sites to facilitate isolation from and integration into these vectors. Particular restriction endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the expression vector utilized. Restriction enzyme sites are chosen so as to properly orient the atrD-encoding DNA with the control sequences to achieve proper in-frame transcription and translation of the atrD molecule. The atrD-encoding DNA must be positioned so as to be in proper reading frame with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which atrD is to be expressed.

Expression of atrD in fungal cells, such as *Saccharomyces cerevisiae* is preferred. Suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD (ATCC 53231) and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 (ATCC 39532)), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 (ATCC 57090, 57091)), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Inducible yeast promoters have the additional advantage of transcription controlled by growth conditions. Such promoters include the promoter regions for alcohol

dehydrogenase 2, isocytochrome C, acid phosphotase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV (ATCC 39475), United States Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization (GAL1 found on plasmid pRY121 (ATCC 37658) and on plasmid pPST5, described below). Suitable vectors and promoters for use in yeast expression are further described by R. Hitzeman et al., in European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal enhancer from *Saccharomyces cerevisiae* (found in conjunction with the CYC1 promoter on plasmid YEpsec--hI1beta, ATCC 67024), also are advantageously used with yeast promoters.

A variety of expression vectors useful in the present invention are well known in the art. For expression in *Saccharomyces*, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb *et al.*, 1979, *Nature* 282:39; Kingsman *et al.*, 1979, *Gene* 7:141 ; Tschemper et al., 1980, *Gene* 10:157) is commonly used. This plasmid contains the *trp* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC 44076 or PEP4-1 (Jones, 1977, *Genetics* 85:12).

Expression vectors useful in the expression of atrD can be constructed by a number of methods. For example, the cDNA sequence encoding atrD can be synthesized using DNA synthesis techniques such as those described above. Such synthetic DNA can be synthesized to contain cohesive ends that allow facile cloning into an appropriately digested expression vector. For example, the cDNA encoding atrD can be synthesized to contain *Not*I cohesive ends. Such a synthetic DNA fragment can be ligated into a *Not*I-digested expression vector such as pYES-2 (Invitrogen Corp., San Diego CA 92121).

An expression vector can also be constructed in the following manner. Logarithmic phase *Aspergillus nidulans*

cells are disrupted by grinding under liquid nitrogen according to the procedure of Minuth *et al.*, 1982 (*Current Genetics* 5:227-231). *Aspergillus nidulans* mRNA is preferably isolated from the disrupted cells using the QuickPrep® mRNA Purification Kit (Pharmacia Biotech) according to the instructions of the manufacturer. cDNA is produced from the isolated mRNA using the TimeSaver® cDNA Synthesis Kit (Pharmacia Biotech) using oligo (dT) according to the procedure described by the manufacturer. In this process an *Eco*RI/*Not*I adapter (Stratagene, Inc.) is ligated to each end of the double stranded cDNA. The adapter modified cDNA is ligated into the vector Lambda Zap^{R}II® using the Predigested Lambda Zap^{R}II®/*Eco*RI/CIAP Cloning Kit (Stratagene, Inc.) according to the instructions of the manufacturer to create a cDNA library.

The library is screened for full-length cDNA encoding atrD using a ^{32}P-radiolabeled fragment of the atrD gene. In this manner, a full-length cDNA clone is recovered from the *Aspergillus nidulans* cDNA library. A full-length cDNA clone recovered from the library is removed from the Lambda Zap^{R}II® vector by digestion with the restriction endonuclease *Not*I which produces a DNA fragment encoding atrD. The atrD encoding fragment is subcloned into plasmid pYES2 for expression studies. In this plasmid the atrD gene is operably linked to the *Saccharomyces cerevisiae GAL*1 promoter at the 5' end, and the yeast *cyc*1 transcription terminator at the 3' end. This plasmid further comprises the ColE1 origin of replication (ColE1) which allows replication in *Escherichia coli* host cells, and the ampicillin resistance gene (Amp) for selection of *E. coli* cells transformed with the plasmid grown in the presence of ampicillin. The expression plasmid further comprises the yeast 2μ origin of replication (2μ ori) allowing replication in yeast host cells, the yeast URA3 gene for selection of *S. cerevisiae* cells transformed with the plasmid grown in a

medium lacking uracil, and the origin of replication from the f1 filamentous phage.

In a preferred embodiment of the invention *Saccharomyces cerevisiae* INVSc1 or INVSc2 cells (Invitrogen Corp., Sorrento Valley Blvd., San Diego CA 92121) are employed as host cells, but numerous other cell lines are available for this use. The transformed host cells are plated on an appropriate medium under selective pressure (minimal medium lacking uracil). The cultures are then incubated for a time and temperature appropriate to the host cell line employed.

The techniques involved in the transformation of yeast cells such as *Saccharomyces cerevisiae* cells are well known in the art and may be found in such general references as Ausubel *et al.*, *Current Protocols in Molecular Biology* (1989), John Wiley & Sons, New York, NY and supplements. The precise conditions under which the transformed yeast cells are cultured is dependent upon the nature of the yeast host cell line and the vectors employed.

Nucleic acid, either RNA or DNA, which encodes atrD, or a portion thereof, is also useful in producing nucleic acid molecules useful in diagnostic assays for the detection of atrD mRNA, atrD cDNA, or atrD genomic DNA. Further, nucleic acid, either RNA or DNA, which does not encode atrD, but which nonetheless is capable of hybridizing with atrD-encoding DNA or RNA is also useful in such diagnostic assays. These nucleic acid molecules may be covalently labeled by known methods with a detectable moiety such as a fluorescent group, a radioactive atom or a chemiluminescent group. The labeled nucleic acid is then used in conventional hybridization assays, such as Southern or Northern hybridization assays, or polymerase chain reaction assays (PCR), to identify hybridizing DNA, cDNA, or RNA molecules. PCR assays may also be performed using unlabeled nucleic acid molecules. Such assays may be employed to identify atrD vectors and transformants and in *in vitro*

diagnosis to detect atrD-like mRNA, cDNA, or genomic DNA from other organisms.

United States Patent Application Serial. No. 08/111680, the entire contents of which are hereby incorporated herein by reference, describes the use of combination therapy involving an antifungal agent possessing a proven spectrum of activity, with a fungal MDR inhibitor to treat fungal infections. This combination therapy approach enables an extension of the spectrum of antifungal activity for a given antifungal compound which previously had only demonstrated limited clinically relevant antifungal activity. Similarly, compounds with demonstrated antifungal activity can also be potentiated by a fungal MDR inhibitor such that the antifungal activity of these compounds is extended to previously resistant species. To identify compounds useful in such combination therapy the present invention provides an assay method for identifying compounds with *Aspergillus nidulans* MDR inhibition activity. Host cells that express atrD provide an excellent means for the identification of compounds useful as inhibitors of *Aspergillus nidulans* MDR activity. Generally, the assay utilizes a culture of a yeast cell transformed with a vector which provides expression of atrD. The expression of atrD by the host cell enables the host cell to grow in the presence of an antifungal compound to which the yeast cell is sensitive to in the untransformed state. Thus, the transformed yeast cell culture is grown in the presence of i) an antifungal agent to which the untransformed yeast cell is sensitive, but to which the transformed host cell is resistant, and ii) a compound that is suspected of being an MDR inhibitor. The effect of the suspected MDR inhibitor is measured by testing for the ability of the antifungal compound to inhibit the growth of the transformed yeast cell. Such inhibition will occur if the suspected *Aspergillus nidulans* MDR inhibitor blocks the ability of atrD to prevent the antifungal compound from acting on the yeast cell. An

illustrative example of such an assay is provided in Example 3.

In order to illustrate more fully the operation of this invention, the following examples are provided, but are not to be construed as a limitation on the scope of the invention.

Example 1

Source of the atrD-Encoding Genomic DNA and cDNA of *Aspergillus nidulans*

Genomic DNA encoding atrD, or the corresponding cDNA sequence (presented in SEQ ID NO:1), may be from a natural sequence, a synthetic source or a combination of both ("semi-synthetic sequence"). The *in vitro* or *in vivo* transcription and translation of these sequences results in the production of atrD. Synthetic and semi-synthetic sequences encoding atrD may be constructed by techniques well known in the art. *See* Brown et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., 68:109-151. atrD-encoding DNA, or portions thereof, may be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A, 380B, 384 or 3848 DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). The polymerase chain reaction is especially useful in generating these DNA sequences. PCR primers are constructed which include the translational start (ATG) and translational stop codon (TAG) of atrD. Restriction enzyme sites may be included on these PCR primers outside of the atrD coding region to facilitate rapid cloning into expression vectors. *Aspergillus nidulans* genomic DNA is used as the PCR template for synthesis of atrD including introns which is useful for expression studies in closely related fungi. In contrast, cDNA is used as the PCR template for synthesis of atrD devoid of introns which is useful for expression in foreign

hosts such as *Saccharomyces cerevisiae* or bacterial hosts such as *Escherichia coli*.

Example 2

Expression of the atrD Protein

Saccharomyces cerevisiae INVSc1 cells (Invitrogen Corp., San Diego CA 92191) are transformed with the plasmid containing atrD by the technique described by J. D. Beggs, 1988, *Nature* 275:104-109). The transformed yeast cells are grown in a broth medium containing YNB/CSM-Ura/raf (YNB/CSM-Ura [Yeast Nitrogen Base (Difco Laboratories, Detroit, MI) supplemented with CSM-URA (Bio 101, Inc.)] supplemented with 4% raffinose) at 28°C in a shaker incubator until the culture is saturated. To induce expression of atrD, a portion of the culture is used to inoculate a flask containing YNB/CSM-Ura medium supplemented with 2% galactose (YNB/CSM-Ura/gal) rather than raffinose as the sole carbon source. The inoculated flask is incubated at 28°C for about 16 hours.

Example 3

Antifungal Potentiator Assay

Approximately 1 x 10^6 cells of a *Saccharomyces cerevisiae* INVSc1 culture expressing atrD are delivered to each of several agar plates containing YNB/CSM-Ura/gal. The agar surface is allowed to dry in a biohazard hood.

An antifungal compound that the untransformed yeast cell is typically sensitive to is dissolved in an appropriate solvent at a concentration that is biologically effective. Twenty μl of the solution is delivered to an antibiotic susceptibility test disc (Difco Laboratories, Detroit, MI). After addition of the antifungal solution the disc is allowed to air dry in a biohazard hood. When dry, the disc is placed on the surface of the petri plates

containing the transformed *Saccharomyces cerevisiae* INVSc1 cells.

Compounds to be tested for the ability to inhibit atrD are dissolved in dimethylsulfoxide (DMSO). The amount of compound added to the DMSO depends on the solubility of the individual compound to be tested. Twenty µl of the suspensions containing a compound to be tested are delivered to an antibiotic susceptibility test disc (Difco Laboratories, Detroit, MI). The disc is then placed on the surface of the dried petri plates containing the transformed *Saccharomyces cerevisiae* INVSc1 cells approximately 2 cm from the antifungal-containing disc. Petri plates containing the two discs are incubated at 28°C for about 16-48 hours.

Following this incubation period, the petri plates are examined for zones of growth inhibition around the discs. A zone of growth inhibition near the antifungal disc on the test plate indicates that the compound being tested for MDR inhibition activity blocks the activity of atrD and allows the antifungal compound to inhibit the growth of the yeast host cell. Such compounds are said to possess MDR inhibition activity. Little or no zone of growth inhibition indicates that the test compound does not block MDR activity and, thus, atrD is allowed to act upon the antifungal compound to prevent its activity upon the host cell.

Example 4

Screen For Novel Antifungal Compounds

A plasmid molecule is constructed which contains DNA sequence information required for replication and genetic transformation in *E. coli* (*e.g.* ampicillin resistance). The plasmid also comprises DNA sequences encoding a marker for selection in fungal cells (*e.g.* hygromycin B phosphotransferase, phleomycin resistance, G418 resistance) under the control of an *A. nidulans* promoter. Additionally,

the plasmid contains an internal portion of the atrD gene (*e.g.* about 3000 base pairs which lack 500 base pairs at the N-terminal end, and about 500 base pairs at the C-terminal end of the coding region specified by SEQ ID NO:1). The atrD gene fragment enables a single crossover gene disruption when transformed or otherwise introduced into *A. nidulans*.

Alternatively, a 5 kilobase pair to 6 kilobase pair region of *A. nidulans* genomic DNA containing the atrD gene is subcloned into the aforementioned plasmid. Then, a central portion of the atrD gene is removed and replaced with a selectable marker, such as hyromycin B phosphotransferase, for a double crossover gene replacement.

Gene disruption and gene replacement procedures for *A. nidulans* are well known in the art (*See e.g.* May *et al*, *J. Cell Biol*. 101, 712, 1985; Jones and Sealy-Lewis, *Curr. Genet*. 17, 81, 1990). Transformants are recovered on an appropriate selection medium, for example, hygromycin (if hygromycin B gene is used in the construction of disruption cassette). Gene replacement, or gene disruption, is verified by any suitable method, for example, by Southern blot hybridization.

Gene disruption or gene replacement strains are rendered hypersensitive to antifungal compounds, and are useful in screens for new antifungal compounds in whole cell growth inhibition studies.